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An Antiviral Agent (46NW-04A) Produced by *Pseudomonas* sp. and Its Activity against Fish Viruses

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Abstract.—The antiviral agent 46NW-04A was isolated and characterized from cell-free culture fluid of *Pseudomonas* sp. 46NW-04 isolated from the aquatic environment. Production of the antiviral substance was maximal at 25°C during days 2–3 of bacterial incubation. Extraction from 30 L of culture fluid by ethyl acetate and purification by thin-layer chromatography on silica gel resulted in 709 mg of the purified antiviral material. Molecular weight of this substance was 1,126 by secondary ionization mass spectrometry, and chemical properties suggested that 46NW-04A was a peptide. Its antiviral activity, measured as the concentration causing 100% plaque reduction, was 25 μg/mL against *Oncorhynchus masou* virus and infectious hematopoietic necrosis virus. However, no antiviral activity was observed against infectious pancreatic necrosis virus at the concentrations tested. *Pseudomonas* sp. 46NW-04 was identified as *Pseudomonas fluorescens* biovar I.

The survival and interaction of viruses and other microorganisms in aquatic environments are important areas for study. Several reports have described the inactivation of viruses in natural water and activated sludges. This antiviral activity seemed to be related to microorganisms inherent in these environments (Magnusson et al. 1967; Fujioka et al. 1980; Toranzo et al. 1982, 1983; Ward 1982; Ward et al. 1986; Knowlton and Ward 1987). It has been reported that this inactivation was caused by proteolytic enzymes produced by bacteria (Cliver and Herrmann 1972; Toranzo et al. 1982, 1983; Ward et al. 1986; Knowlton and Ward 1987). These proteolytic enzymes were not characterized or isolated from the bacteria, although some properties were studied by heat treatment, dialysis, and release by solubilizing agents.

Previously, we investigated the antiviral activity of microorganisms in the hydrosphere (Kamei et al. 1987a, 1987b, 1988a 1988b). In a series of studies on the aquatic microbial ecosystem, we reported the inactivation of fish viruses by natural fresh, brackish, and seawaters. This inactivation appears to be principally caused by bacteria in the water (Kamei et al. 1987a, 1988a). A large number of bacteria were screened from the aquatic environments and some, such as species of *Pseudomonas, Achromobacter,* and *Vibrio,* were found to have antiviral properties (Kamei et al. 1987b, 1988b). *Pseudomonas* sp. 46NW-04 was isolated from the water of the Nanae Fish Culture Experimental Station of Hokkaido University in Hokkaido, Japan. This bacterium produces a substance with potent antiviral activity, which was detected in culture fluid and is effective against infectious hematopoietic necrosis virus (IHNV; Kamei et al. 1988b).

In this study, we isolated and characterized the anti-IHNV agent 46NW-04A from supernatants of cultured *Pseudomonas* sp. 46NW-04. We also conducted a taxonomic comparison of *Pseudomonas* sp. 46NW-04 with seven reference species of *Pseudomonas* by using Bergey's Manual of Systematic Bacteriology (Palleroni 1984).

Methods

Cell cultures.—Gonad cells from rainbow trout *Oncorhynchus mykiss* (RTG-2; Wolf and Quimby 1962) were used for propagation of viruses, and embryo cells from chinook salmon *Oncorhynchus tshawytscha* (CHSE-214; Fryer et al. 1965) were used for plaque assay. For the plaque assay, cells were grown at 15°C in MEM–10–tris medium composed of Eagle's minimum essential medium (MEM, GIBCO), 10% fetal bovine serum (M.A. Bioproduct), 0.075% $NaHCO_3$, 100 international units penicillin/mL (Sigma), 100 μg streptomycin/mL (Sigma), and 1.6% tris buffer [tris(hydroxymethyl)aminomethane–(tris)hydrochloride] (Sigma) adjusted to pH 7.8. According to the method of Kamei et al. (1987c), the cells were suspended in growth medium and seeded in 24-well plates (16-mm-diameter wells, Falcon) to give approximately 10^6 cells/mL in the wells. After 1 d, the monolayers were used for the plaque assay.

Viruses.—The IHNV (strain ChAb) and *Oncorhynchus masou* virus (OMV; strain 00-7812) were

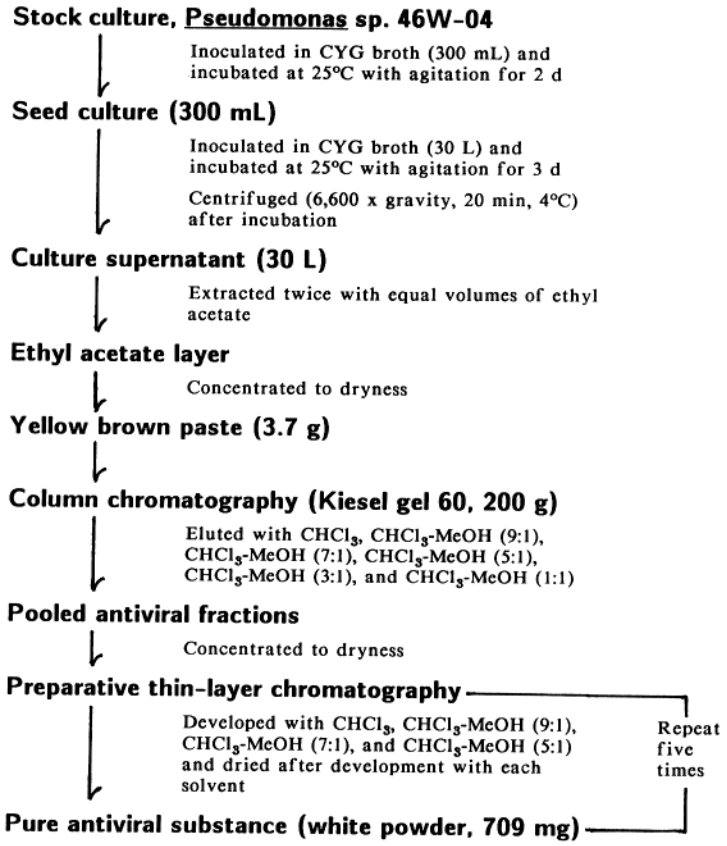

FIGURE 1.—Outline of procedure for isolation of antiviral substance 46NW-04A. CYG = casamino acid–yeast extract–glucose broth.

isolated in our laboratory from chum salmon *Oncorhynchus keta* and masu salmon *O. masou*, respectively. Infectious pancreatic necrosis virus (IPNV; strain VR299) was provided by J. L. Fryer (Oregon State University). These stock viruses were propagated at 15°C with RTG-2 cells in 75-cm^2 tissue culture flasks (Falcon) containing 25 mL of MEM–10–tris medium. When the cytopathic effect was complete, the culture fluid was removed from the flasks and clarified by centrifugation at 2,100 × gravity at 4°C for 20 min. The supernatants were filtered through a 0.40-μm-pore filter (Nuclepore) for OMV and 0.45-μm-pore filter (Millex-HA, Millipore) for IHNV and IPNV. The titers of IHNV, IPNV, and OMV that yielded a tissue culture infective dose per milliliter causing a cytopathic effect in 50% of cultures inoculated (TCID50/mL) were $10^{5.8}$, $10^{8.3}$, and $10^{4.3}$, respectively. The viruses were stored at −80°C in 2.0-mL aliquots until used.

Plaque assay.—Production of antiviral substances by bacteria was ascertained by plaque-reduction assay with IHNV (Kamei et al. 1987c, 1988b). Purified 46NW-04A was dissolved in dimethylsulfoxide (DMSO) and diluted with Hanks' balanced salt solution (Hanks' BSS, GIBCO) to make a stock solution of 1 mg/mL. The stock solution was diluted to desired concentrations with Hanks' BSS containing 1% DMSO. A 0.2-mL aliquot of the dilution was mixed with an equal volume of IHNV suspension containing approximately 150 plaque-forming units (PFU)/0.1 mL and incubated at 15°C for 1 h. The control fluid was 1% DMSO in Hanks' BSS.

Determination of optimal conditions for 46NW-04A production.—Determination of optimum incubation temperature for *Pseudomonas* sp. 46NW-04 was done by inoculating a loopful of seed culture (5.1×10^9 colony-forming units (CFU)/mL) into 15 mL of casamino acid–yeast extract–glucose (CYG) broth (Kamei et al. 1988b) in L-form test tubes and incubating these with agitation at temperatures ranging from 10 to 33°C for 2 d with a Temperature Gradient Bio-photorecorder (TN-

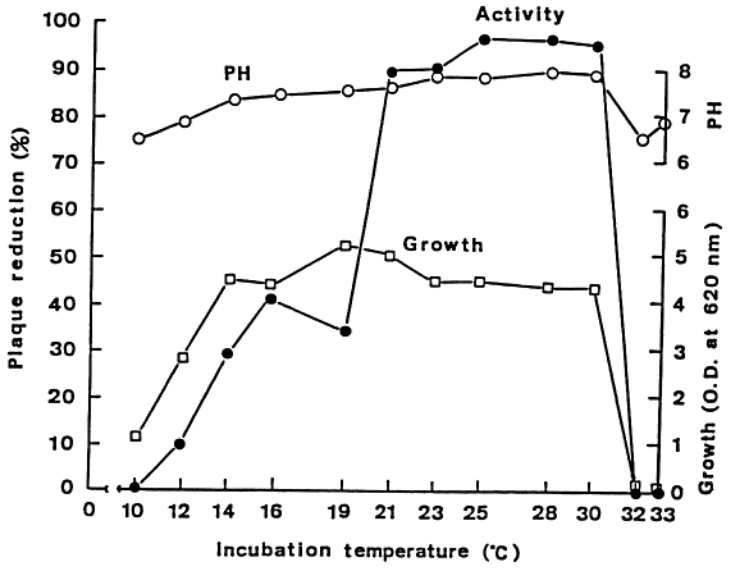

FIGURE 2.—Effect of incubation temperature on growth and production of anti-IHNV agent by *Pseudomonas* sp. 46NW-04; O.D. = optical density.

112D, Toyo Kagaku Sangyo Co., Ltd.). After incubation, the pH and optical density at 620 nm were measured, and the culture broth was tested for antiviral activity by plaque-reduction assay. For the determination of the rate of production of the antiviral substance, the bacterium was grown in 400 mL of CYG broth in a 500-mL Sakaguchi flask and incubated with agitation at 25°C for 7 d. At 12-h intervals, 5-mL aliquots of the culture were aseptically withdrawn, and the pH was measured. Antiviral activity then was determined by plaque reduction.

Isolation and purification of antiviral substance 46NW-04A.—One loopful of stock culture of *Pseudomonas* sp. 46NW-04 was inoculated in 300 mL of CYG broth in a 500-mL Sakaguchi flask and incubated with agitation at 160 revolutions per minute for 2 d at 25°C. Four 2.5-L CYG broths in Erlenmeyer flasks were each inoculated with 25 mL of broth culture and incubated with agitation at 25°C for 3 d. After incubation, the spent culture was centrifuged at 6,600 × gravity at 4°C for 20 min. The supernatant was extracted twice with an equal volume of ethyl acetate, and the ethyl acetate layer was concentrated to 2 L under vacuum at 25°C. The concentrated solvent phase was dehydrated with Na_2SO_4 (anhydrous) and evaporated to dryness. This extraction was conducted three times to obtain extracts from 30 L of culture fluid (Figure 1). Detection of 46NW-04A was carried out with anisaldehyde reagent (Stahl and Kaltenbach 1961).

Chemical analysis.—Solubility of 46NW-04A in solvent was tested with acetone, benzene, chloroform, diethyl ether, DMSO, ethanol, *n*-hexane, methanol, pyridine, ethyl acetate, petroleum ether, and water. The color reaction was tested with anisaldehyde, anthrone, Barton solution, bromcresol green, dinitrophenylhydrazine, Dragendorff solution, 8-hydroxyquinoline-NH_3, copper acetate, Legal solution, Molish solution, ninhydrin, rhodamine, and potassium permanganate.

Melting point was measured with a Micro Melting Point Apparatus (Yanako). Optical rotation was performed with an AA-5 spectrometer (Optical Activity Ltd.). Ultraviolet spectrum was recorded with a 150-20 spectrophotometer (Hitachi). Infrared spectrum was recorded with an A-100 spectrophotometer (Japan Spectrascopic Co., Ltd.). Secondary ionization mass measurement was supplied by Ueno Fine Chemical Industries, Ltd.

Antiviral activity of 46NW-04A against salmonid viruses.—Antiviral activity of 46NW-04A was tested against two RNA viruses—IHNV and IPNV—and the DNA virus OMV. Serial twofold dilutions of the substance were made in 1% DMSO in Hanks' BSS to give the concentrations of 100, 50, 25, 12.5, and 6 μg/mL. These were compared for antiviral activity by plaque reduction.

Taxonomic study of Pseudomonas sp. 46NW-04.—Tests used to classify *Pseudomonas* sp. 46NW-04 were those of Bergey's Manual of Systematic Bacteriology (Palleroni 1984) and Stanier

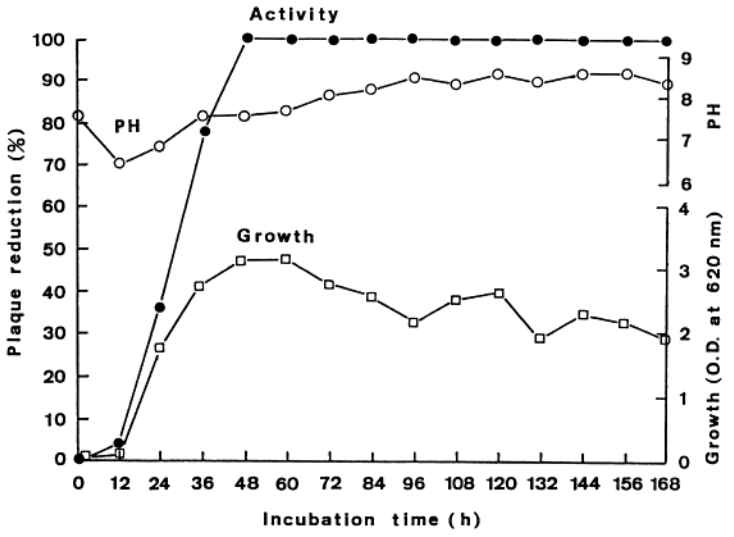

FIGURE 3.—Time course of growth and the production of anti-IHNV agent by *Pseudomonas* sp. 46NW-04 incubated at 25°C; O.D. = optical density.

et al. (1966). The tests included seven reference strains of *Pseudomonas*: *Pseudomonas fluorescens* (National Collection of Marine Bacteria: NCMB 129), *P. putida* (NCMB 406), *P. stutzeri* (American Type Culture Collection: ATCC 17588), *P. mendocina* (ATCC 25411), *P. pseudoalcaligenes* (ATCC 17440), *P. acidovorans* (ATCC 15668), and *P. saccharophila* (Institute of Applied Microbiology, Tokyo University: IAM 1504). Freshwater agar (FWA) medium (Yoshimizu et al. 1976) was used as the basal medium for tests of gelatin, starch, Tween 80, and poly-β-hydroxybutyrate (PHB) hydrolysis, levan formation, and denitrification. The test strains were grown in 5 mL of trypticase soy broth (TSB; Baltimore Biological Laboratory) at 25°C for 24 h. One loopful of each culture was inoculated into each of the test media. Each test was read after incubation at 25°C for 5 d. The percent of guanine and cytosine in the bacterial DNA (mol % GC) was measured and averaged for three trials.

Results

Production of 46NW-04A

Production of the antiviral agent 46NW-04A by *Pseudomonas* sp. 46NW-04 was first determined at temperatures from 10 to 33°C. The bacterium grew well between 14 and 30°C, and maximum growth was observed at 19°C. Antiviral activity was highest in the culture incubated at 25–30°C (Figure 2). During the incubation period, the pH changed from 7.2 to about 7.8, and this change did not affect the antiviral activity. Production of

TABLE 1.—Physicochemical properties of antiviral substance 46NW-04A produced by *Pseudomonas* sp. 46NW-04.

Characteristic	Description or value
Appearance	White powder
Melting point	206–208°C
Optical rotation at 25°C (2% solution in methanol)	−47.5
Molecular weight (by mass spectrometry)	1,126
Elemental analysis	C, 54.68%; H, 8.49%; N, 10.0%
Wavelength (in nm) at maximum absorption of ultraviolet light (molecular extinction coefficient)	204 (18,200)
Absorption bands (cm^{-1}) of infrared light	3,300, 2,950, 2,920, 2,850, 1,730, 1,650, 1,520, 1,460, 1,370, 1,270, 1,060
Stability	Stable at pH 3–10
Solubility	Soluble in methanol, ethanol, ethyl acetate, acetone, benzene, chloroform, diethyl ether, dimethylsulfoxide, and pyridine. Insoluble in *n*-hexane, petroleum ether, and water.
Color reaction	Positive for anisaldehyde, bromcresol green, dinitrophenylhydrazine, Dragendorff solution, and 8-hydroxyquinoline-NH_3 Negative for potassium permanganate, anthron, Barton solution, copper acetate, Legal solution, Molish solution, ninhydrin, and rhodamine

TABLE 2.—Activity of antiviral substance 46NW-04A against fish viruses.

Concentration of 46NW-04A (μg/mL)	Antiviral activity (% plaque reduction) against		
	OMV	IHNV	IPNV
50	100	100	0
25	100	100	0
12.5	100	94	0
6	60	58	0
3	25	37	0

antiviral substance was tested during incubation at 25°C. Bacterial growth reached a maximum at 48 h postincubation and antiviral activity increased during growth reaching a maximum after incubation for 48 h (Figure 3). Antiviral activity against IHNV remained high even after incubation for 168 h.

Purification of 46NW-04A from the Culture Fluid

In the preliminary experiments, antiviral activity was detected only in culture supernatants, but not in the cell pellets. After cultivation with agitation for 3 d at 25°C, the culture had a pH of 8.6. When the antiviral substance was extracted with two volumes of ethyl acetate from 30 L of supernatant without acidification of the culture, about 3.7 g of a yellow-brown paste was produced. Following suspension in a mixture of chloroform and methanol, the material was subjected to column chromatography. Active fractions were pooled, concentrated, and further purified by thin-layer chromatography (TLC). Finally, 709 mg of a white powder (46NW-04A) was obtained from an original volume of 30 L of culture. This substance was not detected on a TLC plate under ultraviolet light; therefore, purity was confirmed by anisaldehyde color reaction as a single spot on a TLC plate developed with various solvent sytems.

Physicochemical Properties of 46NW-04A

Physicochemical properties of 46NW-04A are summarized in Table 1. This substance was readily soluble in acetone, methanol, ethanol, and

TABLE 3.—General characteristics of strains 46NW-04 and NCMB 129 of *Pseudomonas fluorescens* and species 1–5 of *Pseudomonas* (section I: *P. fluorescens* biovars I–V, *P. aeruginosa, P. chlororaphis, P. aureofaciens,* and *P. putida* biovars A and B) listed in Bergey's Manual of Systematic Bacteriology (Palleroni 1984). Abbreviations: *P. a.* = *Pseudomonas aeruginosa*; *P. ch.* = *P. chlororaphis*; *P. au.* = *P. aureofaciens*; + = positive reaction; − = negative reaction; d = reaction depends on bacterial strain; mol % GC = the percent of guanine and cytosine in the bacterial DNA.

Characteristics	46NW-04	Reference species					
		NCMB 129	*P. fluorescens* Biovar				
			I	II	III	IV	V
(1) Cell diameter, μm	0.9		0.7–0.8	0.7–0.8	0.8	0.7	0.8
(2) Cell length, μm	2.2		2.3–2.8	2.0–2.8	2.0–2.8	2.0–2.5	2.0–3.0
(3) Number of flagella	>1		>1	>1	>1	>1	>1
(4) Pyocyanin production	−	−	−	−	−	−	−
(5) Pyoverdin production	+	+	+	d	+	+	d
(6) Chlororaphin production	−	−	−	−	−	−	−
(7) Phenazine monocarboxylate production	−	−	−	−	−	−	−
(8) Other pigments (not carotenoids)	−	−	−	−	−	d	−
(9) Yellow-orange cellular pigments	−	−	−	−	−	−	−
(10) Oxidase	+	+	+	+	+	+	+
(11) Poly-β-hydroxybutyrate (PHB) accumulation	−	−	−	−	−	−	−
(12) Levan formation from sucrose	+	+	+	+	−	+	−
(13) Gelatin liquefaction	+	+	+	+	+	+	+
(14) Starch hydrolysis	−	−	−	−	−	−	−
(15) Autotrophic growth with H_2			−	−	−	−	−
(16) Lecithinase (egg yolk)	+	+	+	±	+	+	d
(17) Lipase (Tween 80 hydrolysis)	−	−	d	−	d	d	d
(18) Extracellular PHB hydrolysis	−	−	−	−	−	−	−
(19) Growth at 4°C	+	+	+	+	+	+	d
(20) Growth at 41°C	−	−	−	−	−	−	−
(21) Denitrification	−	−	−	+	+	+	−
(22) Arginine dihydrolase	+	+	+	+	+	+	+
(23) Catechol, ortho cleavage			+	+	+	+	+
(24 Protocatechuate, ortho cleavage	+	+	+	+	+	+	+
(25) Mol % GC	59.9	60.7	60.5	61.3	60.6	59.4	60.5

DMSO; weakly soluble in benzene, chloroform, ethyl acetate, and pyridine; very weakly soluble in diethyl ether; and insoluble in *n*-hexane, petroleum ether, and water. Color reactions of 46NW-04A were positive for anisaldehyde, bromcresol green, dinitrophenylhydrazine, Dragendorff solution, and 8-hydroxyquinoline-NH_3, but negative for anthron, Barton solution, copper acetate, Legal solution, Molish solution (in this test, green color occurred), ninhydrin, potassium permanganate, and rhodamine.

Antiviral substance 46NW-04A is a white powder with a melting point of 206–208°C. A 2% solution of 46NW-04A in methanol has an optical rotation of −47.5° at 25°C. Its main constituents are carbon (54.68%), hydrogen (8.49%), and nitrogen (10.0%). Its absorption of ultraviolet light in methanol showed a maximum at 204 nm with a molecular extinction coefficient at this wavelength of 18,200. Molecular weight was determined to be 1,126 by mass spectrometry. The infrared absorption spectrum of 46NW-04A showed bands characteristic of amine (3,300 cm^{-1}), ketone (1,730 and 1,270 cm^{-1}), and carbonyl residues (1,650 cm^{-1}), properties that suggest 46NW-04A is a peptide.

TABLE 3.—Extended.

Characteristics	Reference species				
				P. putida	
	P. a.	*P. ch.*	*P. au.*	Biovar A	Biovar B
(1)	0.5–0.7	0.7–0.8	0.7–0.8	0.7–1.1	0.7–1.1
(2)	1.5–3.0	1.5–3.6	1.9–2.8	2.0–4.0	2.0–4.0
(3)	1	>1	>1	>1	>1
(4)	+	−	−	−	−
(5)	+	d	+	+	d
(6)	−	+	−	−	−
(7)	−	−	+	−	−
(8)	+	−	−	−	−
(9)	−	−	−	−	−
(10)	+	+	+	+	+
(11)	−	−	−	−	−
(12)	−	+	+	−	−
(13)	+	+	+	−	−
(14)	−	−	−	−	−
(15)	−	−	−	−	−
(16)	−	+	d	−	−
(17)	±	+	d	d	d
(18)	−	−	−	−	−
(19)	−	+	+	d	+
(20)	+	−	−	−	−
(21)	+	+	−	−	−
(22)	+	+	+	+	+
(23)	+	+	+	+	+
(24)	+	+	+	+	+
(25)	67.2	63.5	63.6	62.5	60.7

Antiviral Activity against Three Fish Viruses

Antiviral activity of 46NW-04A was examined with both DNA (OMV) and RNA (IHNV and IPNV) viruses. Plaque formation by OMV was completely inhibited by the addition of 12.5 μg 46NW-04A/mL of this agent (Table 2). Even at 6 μg/mL, 60% plaque reduction of the virus was observed, but little or no antiviral activity was noted at 3 μg/mL. Similar results were obtained with IHNV; plaque reduction was 94% at 12.5 μg/mL and 37% when the concentration of this substance decreased to 3 μg/mL. No antiviral activity was observed for IPNV at concentrations as high as 50 μg/mL. These results suggest that 46NW-04A is only effective for enveloped viruses, regardless of their genome, because IHNV and OMV are enveloped viruses, whereas IPNV is not.

Identification of Pseudomonas sp. 46NW-04

We compared the characteristics of *Pseudomonas* sp. 46NW-04 to seven reference species of *Pseudomonas*. The accumulation of PHB, an important characteristic for the differentiation of *Pseudomonas* sp., was negative for *Pseudomonas* sp. 46NW-04, but not for *P. pseudoalcaligenes* and *P. acidovarans*. This suggests that *Pseudomonas* sp. 46NW-04 belongs to section I of this genus according to Bergey's Manual of Systematic Bacteriology (Table 3). The strain could be grouped into any species or biovar of *P. aeruginosa, P. fluorescens, P. chlororaphis, P. aureofaciens,* or *P. putida* because it was positive for pyoverdin production and arginine dihydrolase; however, *Pseudomonas* sp. 46NW-04 was positive for lecithinase and negative for production of pycyanine, chlororaphin, and phenazine monocarboxylate, so it is a strain of *Pseudomonas fluorescens*. In addition, examination of biovars I–V of this species showed that *Pseudomonas* sp. 46NW-04 may be identified as *Pseudomonas fluorescens* biovar I, based on the properties of positive levan formation and negative denitrification (Table 4). The mol % GC of DNA of the strain was 59.9, which confirmed that the *Pseudomonas* sp. that produces antiviral substances should be assigned to *Pseudomonas fluorescens*.

Discussion

We have reported that fish viruses were inactivated in freshwater, brackish water, and seawater samples, and that the inactivation was possibly caused by bacterial metabolites released in the water (Kamei et al. 1987a, 1988a; Yoshimizu et al. 1986). This possibility was investigated by screening about 1,500 bacterial isolates from water

TABLE 4.—Characteristics of strains 46NW-04 and NCMB 129 of *Pseudomonas fluorescens* and species 2 (*P. fluorescens* biovars I–V), 3 (*P. chlororaphis*), and 4 (*P. aureofaciens*) of *Pseudomonas* (section I) listed in Bergey's Manual of Systematic Bacteriology (Palleroni 1984). The d indicates strain dependence.

Characteristics	Reference species								
		P. fluorescens							
			Biovar					*P. chloro-raphis*	*P. aureo-faciens*
	46NW-04	NCMB 129	I	II	III	IV	V		
P. fluorescens biovars as designated by Stanier et al. (1966)			A	B	C	F	G	D	E
Nonfluorescent pigments									
Green (chlororaphin)	−	−	−	−	−	−	−	+	−
Orange (phenazine-1-carboxylate)	−	−	−	−	−	−	−	−	+
Blue, nondiffusible	−	−	−	−	−	+	−	−	−
Levan formation from sucrose	+	+	+	+	−	+	−	+	+
Denitrification	−	−	−	+	+	+	−	+	d
Carbon sources used for growth:									
L-Arabinose	+	+	+	+	d	+	d	−	+
Sucrose	+	+	+	+	−	+	d	+	d
Saccharate	−	−	+	+	d	+	d	+	+
Propionate	+	+	+	+	d	+	+	+	+
Butyrate	+	+	−	d	d	+	d	+	+
Sorbitol	+	+	+	+	d	+	d	−	−
Adonitol	+	+	+	−	d	−	d	−	−
Propylene glycol	+	+	−	+	d	−	d	−	−
Ethanol	−	−	−	+	d	−	d	d	−

and sediments by the plaque-reduction method (Kamei et al. 1987b, 1988b).

In this study, purification and testing of the antiviral substance from the culture supernatant of *Pseudomonas* sp. 46NW-04 was made by extraction with ethyl acetate, column chromatography, and TLC on silica gel. In the first step of the purification, a 50-μg/mL concentration of crude extract from the ethyl acetate layer completely inhibited plaque formation of IHNV (approximately 150 PFU) in CHSE-214 cells. However, antiviral activity was not detected in the bacterial cell pellet treated with methanol. The purified antiviral substance, 46NW-04A, showed potent antiviral activity against IHNV and OMV; it caused 100% plaque reduction at 25 μg/mL and still maintained activity at 12.5 μg/mL. From 30 L of culture broth, 709 mg of the purified substance was recovered, which corresponded to 23 μg/mL in the original culture solution. Our previous study showed that a 10% culture filtrate of *Pseudomonas* sp. (although the strain was different from the strain used in this study) caused considerable decrease of IHNV titer during a 3-d incubation period, and the viral inactivation was dependent on duration of exposure to the culture filtrate. We further demonstrated that a significant loss of viral infectivity (approximately 99%) occurred within 3 d in pure bacterial cultures in 50% artificial seawater when washed cultures were used instead of the culture filtrate (Kamei et al. 1987a).

Several investigators have reported that the lack of viral survival in natural water is caused by microorganisms or extracellular compounds released from microorganisms (Toranzo et al. 1982, 1983; Ward et al. 1986). However, there have been no reports on antiviral substances actually isolated and characterized from microorganisms indigenous to aquatic environments. Several investigators have suggested that the causative agent of viral inactivation was proteolytic bacterial enzymes (Toranzo et al. 1982, 1983; Ward et al. 1986; Knowlton and Ward 1987), but few compounds with antiviral action have been isolated from microorganisms in aquatic environments. Most reports are limited to antibiotics active against bacteria or fungi (Rosenfeld and Zobell 1947; Grein and Meyers 1958; Burkholder et al. 1966; Doggett 1968; Ballester et al. 1977; Wratten et al. 1977; Lemos et al. 1985). Most bacteria that produce antiviral substances are actinomycetes isolated from marine mud (Okazaki and Okami 1972; Okazaki et al. 1975; Okami et al. 1976). *Pseudomonas* and *Vibrio* species have been reported to produce proteolytic enzymes (Toranzo et al. 1982, 1983). However, because of limited

information about antibiotic-producing microorganisms in water, the ecological role of these microbes in the environment is still unknown.

The production of antiviral substance by *Pseudomonas* sp. 46NW-04 was influenced by incubation temperature. The antiviral activity was highest when the bacterium was grown at temperatures between 21 and 30°C, although growth of the bacterium was observed between 14 and 19°C. Neither antiviral activity nor bacterial growth was observed at temperatures higher than 30°C, and the production of antiviral substance was greater at temperatures higher than the optimal growth temperature.

Studies of the time course of production showed that the amount of antiviral substance reached a maximum by 48 h when bacterial cultures were incubated at 25°C and agitated. Activity of the antiviral substance remained high for 168 h. This indicated that the antiviral substance was not metabolized further and can be regarded as a stable substance. We believe this is the first report characterizing an antiviral substance from a *Pseudomonas* sp. isolated from an aquatic environment. This finding suggests the possibility that the antiviral activity observed in aquatic ecosystems may be due to the production of low-molecular-weight substances. The antiviral substance 46NW-04A described in this report was different from the proteolytic enzymes described by Ward et al. (1986) and Knowlton and Ward (1987) because 46NW-04A has a molecular weight of 1,126. Negative ninhydrin reaction suggested that 46NW-04A was a peptide blocked in its *N*-terminal residues.

Some bacterial enzymes may act synergistically by disrupting the viral structural integrity, but the mechanism is not known in detail. Preliminary experiments suggest that the antiviral substance 46NW-04A prevents adsorption of virus particles on host cells by changing the virus morphology or by coating the virus surface. Antiviral effect was found even if the substance was washed from infected cells, and the reactivity was particularly strong against enveloped viruses. Studies of the action of this antiviral substance on the viral structure could serve as a basis for elucidation of the mechanism of attachment and penetration of these fish viruses into host cells.

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